FLUORESCENCE IMAGE ANALYSIS FOR QUANTIFICATION OF ACTIVE OXYGEN INDUCED BY PHOTOCHEMICAL REACTION

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Abstract— **This study was performed to confirm that activated leukocytes are concerned with Vascular Shut Down effect ; VSD in PhotoDynamic Therapy ; PDT. Hydrogen peroxide ; H_2O_2, which is a kind of active oxygen that came from monocytes, was made visibility under the confocal laser scanning microscope ; CLSM, and tried to quantify the amount of its formation from the fluorescent intensity with image analysis. Fluorescence images acquired by CLSM were analyzed, and the fluorescence intensity was expressed as gray level, which is graded from 0 to 255. Only the fluorescence derived from a monocyte added Zinc coproporphyrin III ; ZnCP-III just before measurement and with HeNe laser irradiation caused fluorescence distribution move to increase as the time course, while no change of distribution was especially observed in other three conditions (only added Zn CP-III, only irradiated with HeNe laser, Non-treated). The result indicates that photochemical reaction occurred due to excitation of photosensitizer, and active oxygen derived from the reaction stimulated monocytes. The activated monocytes, this time for themselves, generated active oxygen and H_2O_2 was made visibility by dichlorofluorescin ; DCFH fluorescence method. In conclusion, we confirmed that activated monocytes by photochemical reaction are concerned with VSD effect.**

Keywords— **active oxygen, photochemical reaction, vascular shut down, monocyte, confocal laser scannning microscope**

I. Introduction

Photodynamic therapy ; PDT attracting attention as a new cancer cure recently is what is operated with photosensitizing drug in advance and laser as excitation light. The photosensitizing drug is a substance that causes photochemical reaction by the irradiation of excitation light and leads to the incidence of active oxygen. As a result, the active oxygen selectively attacks the area in which photosensitizing drugs collects.

At the beginning, active oxygen, which occurs due to the photochemical reaction caused in this way, is thought to be the main factor of the cytotoxic effect, which is direct action of PDT. After that, it became clear that the PDT occludes blood vessels in tumors and neovasculatures, and that it blocks the tumorous tissue by tissue ischemia, rather than it attacks the tumorous tissue itself. And it has been called Vascular Shut Down effect ; VSD effect.

We ascertained that a leukocyte cell inside the blood vessel asked to contribute to the VSD effect, with the experiment *in vivo* and intravital microscopy model. In other words, it was showed that as a result of the observation of behavior of leukocytes during the PDT with fluorescent staining and an imageintensified camera, the leukocytes accumulate in the thrombus and have influence on the VSD effect [1]. But, as for the details, it is not clear about how VSD effect occurs especially in a single cell level.

So, monocyte of Wistar rat was separated, and DCFH-DA fluorescence derived from activated single monocyte by photochemical reaction was observed under the confocal laser scanning microscope ; CLSM. In general, this way has been called Dichlorofluorescin (dihydrodichlorofluorescein ;DCFH) fluorescence method and often used but *in vitro* and *in vivo* experiment also diagnosis of phagocytosic function of polymorphonuclear neutrophils ; PMN in pediatrics [2].

In this study, hydrogen peroxide;H_2O_2, which is a kind of monocyte origin active oxygen, was made visibility under CLSM, and tried to quantify the amount of its formation from the fluorescent intensity with image analysis in order to confirm that activated leukocytes are concerned with VSD.

II. Methods

A. Chemicals

Novo Heparin was purchased from Novo Nordisk A/S, Denmark. Nembutal Sodium Solution was purchased from Abbott Laboratories, USA. Lympholyte-Rat (density $= 1.094 \pm 0.001\,\mathrm{g/cm^3}$ at $22\,^\circ\mathrm{C}$) was purchased from Cedalane Laboratories Ltd., Canada. 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate ; DCFH-DA) was purchased from Molecular Probes, Inc., USA. 2',7'-DichloroFluorescein was purchased from Polysciences, Inc., USA. Zinc coproporphyrin III tetrasodium salt (M.W. = 718.094) was purchased from Porphyrin Products, Inc., USA. Hank's Balanced Salt Solution ; HBSS(-), and Phosphate Buffered Salines ; PBS(-) and 1 M Tris-HCl pH 7.5 was purchased from GIBCO BRL, Life Technologies, Inc., USA. Albumin, Human (Fraction V Powder 96-99% Albumin) was purchased from Sigma-Aldrich Co., USA. Ammonium Chloride was purchased from Wako Pure Chemical Industries.Ltd., Japan.

B. Cell Preparation

Monocytes were prepared from the blood of male Wistar rat (Sankyou lab. Service Corporation Inc., Tokyo, from 9 to 13-weeks-old, weighing about 300 g) by means of density gradient separation.

Peripheral blood monocytes were isolated from heparinized caval blood of adult Wistar male rat under anesthesia of 1.0 ml/kg Nembutal, and the extracted blood was layered over Lymphocyte-Rat carefully as little mixing as possible at the interface. After centrifugation for 30 minutes at 1500 rpm at room temperature, well-defined white and turbid layer at the interface was removed and

Report Documentation Page

Report Date	Report Type	Dates Covered (from... to)
25 Oct 2001	N/A	-

Title and Subtitle Fluorescence Image Analysis for Quantification of Active Oxygen Induced by Photochemical Reaction	**Contract Number**
	Grant Number
	Program Element Number
Author(s)	**Project Number**
	Task Number
	Work Unit Number
Performing Organization Name(s) and Address(es) Institute of Applied Physics and Physico-Informatics Graduate School of Science and Technology Keio University, Yokohama, Japan	**Performing Organization Report Number**
Sponsoring/Monitoring Agency Name(s) and Address(es) US Army Research, Development & Standardization Group (UK) PSC 802 Box 15 FPO AE 09499-1500	**Sponsor/Monitor's Acronym(s)**
	Sponsor/Monitor's Report Number(s)

Distribution/Availability Statement
Approved for public release, distribution unlimited

Supplementary Notes
Papers from 23rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society, October 25-28, 2001, held in Istanbul, Turkey. See also ADM001351 for entire conference on cd-rom.

Abstract

Subject Terms

Report Classification unclassified	**Classification of this page** unclassified
Classification of Abstract unclassified	**Limitation of Abstract** UU

Number of Pages
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transferred to a new centrifuge tube. After centrifugation for 10 minutes at 1500 rpm, lysis of erythrocytes was performed with lysis buffer at 37°C for 10 minutes. The lysis buffer consisted of 13.5 ml of 0.83% Ammonium Chloride and 1.5 ml of 0.2 M Tris-HCl (pH 7.5) and 75μl of 1% Albumin and they were freshly mixed out of necessity. And after wash once with PBS (-), 6-8 $\times 10^6$ cells/ml the monocytes was suspended in HBSS (-) on ice until they were needed.

C. Protocol

DCFH-DA was added to the cell suspension at a final concentration of 5 μM, they were incubated at 37 °C for 5 minutes. Zn CP-III was added to the suspension at a final concentration of 20 μg/ml. About 10 μl of the cell suspension was dropped onto a clean microscope slide and a cover slip applied. Excess fluid was expressed and cells were observed by CLSM. Both differential interference contrast ; DIC and fluorescence images were obtained. The Zn CP-III concentration reflects the result we obtained from experiments *in vivo*. In consequence of the experiment in vivo, it was conformed that the photosensityzer reaches the peak of concen-tration of Zn CP-III in blood just after its administration, and the quantity becomes about 50 μ g/ml. At about 2 hours after the administration, it comes to the half concentration, and then gradually the excretion graduates, and at 24 hours after, there is little Zn CP-III in the plasma. So, we chose concentration of 20 μ g/ml at about 3 hours after administration because it was the almost least concentration that VSD effect occurs efficiently and the rate of occlu-tion was near 100 % .

D. Confocal Laser Scanning Microscopy

Determination of cellular uptake and localization was based on fluorometric assay [3]. Cellular fluorescence was acquired using CLSM (LSM410, Carl Zeiss Co., Germany) equipped with a 63 × objective lens (NA 1.4).

To detect DCF fluorescence, a blue Ar laser ($\lambda = 488$ nm) and a 515 nm long pass filter were used for excitation and emission respectively every a minute. The power of Ar laser was 0.15 mW and irradiation continued for 5.77 seconds. And except irradiation of Ar laser, a green HeNe laser ($\lambda = 543$ nm) was used for excitation of Zn CP-III to cause photochemical reaction. The power of HeNe laser was 0.04 mW.

David A. Bass et. al. showed excitation and emission spectrum of reagent DCF and of PMNL preincubated with DCFH-DA and stimulated by phorbol myristate acetate ; PMA [2]. Their maximal excitation and emission wavelength were 504 and 526 nm, respectively.

Nagao et al. showed the absorption and fluorescence spectra of ZnCP-III [4].

The absorption spectra of ZnCP-III had three peaks, those were 404,536,572 nm. The second highest peak at 536 nm was decided to be used in this measurement. Using the peak at 536 nm of the absorption spectrum for excitation of ZnCP-III, the excitation spectrum of DCF do not overlap it because excitation wavelength of DCF is in the range 470-510 nm.

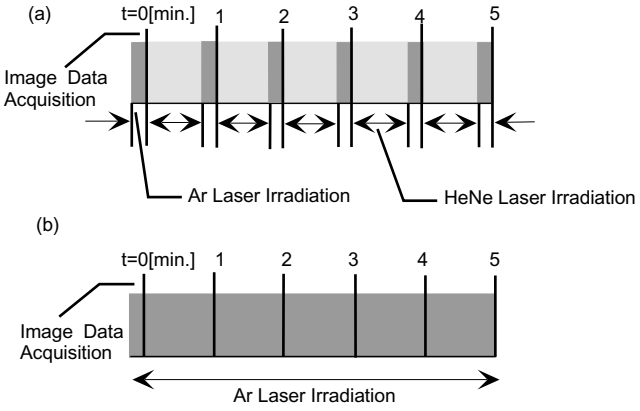

Fig. 1. The protocol of Ar and HeNe laser irradiation. (a) Monocytes were irradiated with Ar laser to detect DCF fluorescence every a minute for five minutes. And at the other moment, they were irradiated with HeNe laser to excite Zn CP-III in order to cause photochemical reaction. (b) Monocytes were irradiated with Ar laser for five minutes continuously, but image datas were acquired every a minute similar to the above.

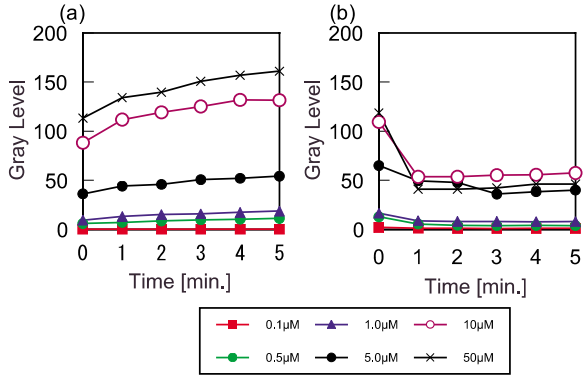

Fig. 2. Evaluation of photobleaching of DCF by difference of Ar laser irradiation. DCF solution of the above six kind of concentration were used, and the way of irradiation of Ar laser was following the protocol given in Fig. 1, except the fact that HeNe laser was not irradiated in this measurement.

The observation was performed under conditions that satisfy a linear correlation between the concentration of DCF and the obtained fluorescence intensity.

In addition, there is a general problem called photobleaching when fluorescence intensity is measured. Actually, the photobleaching of a photosensitizing drug is also a serious problem as PDT treatment is performed. Fig. 1 shows the protocol of irradiation of Ar and HeNe laser. Fig. 1 (a) shows that monocytes were irradiated with Ar laser to detect DCF fluorescence every a minute for five minutes. And at the other moment, they were irradiated with HeNe laser to excite Zn CP-III in order to cause photochemical reaction. Whereas, Fig. 1 (b) shows that monocytes were irradiated with Ar laser for five minutes continuously, but image datas were acquired every a minute similar to the above.

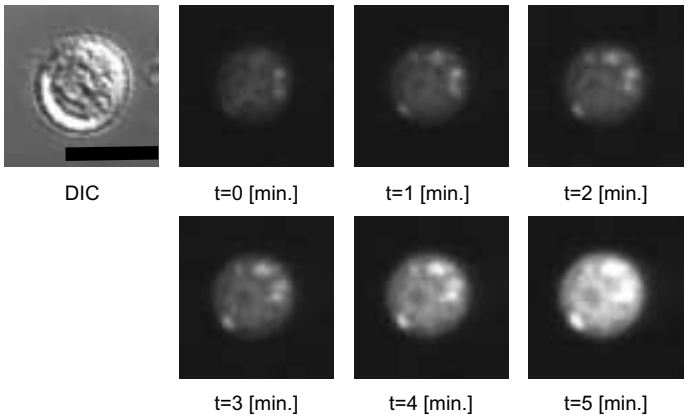

Fig. 3. DIC and fluorescence images acquired by CLSM. 6-8×10^6 cells/ml monocytes were suspended in HBSS (-), and incubated with 5 μM DCFH-DA for 5 minutes at 37 °C. Then 20 μg/ml ZnCP-III was added and the cells were observed under CLSM. Bar=10 μm.

III. Results

A. Photobleaching characteristics

Photobleaching characteristics of DCF fluorescence are given in Fig. 2. DCF solution of six kind of concentration, those are 0.1 0.5, 1.0, 5.0, 10, 50 μ M, were used. The way of irradiation of Ar laser in Fig. 2 (a) and (b) was following the protocol given in Fig. 1 (a) and (b) respectively, except the fact that HeNe laser was not irradiated in this measurement.

Continuous irradiation for 5 minutes of Ar laser caused photobleaching, that is to say, reduction of DCF fluorescence intensity obviously, while irradiation of Ar laser every a minute did not cause definite reduction of fluorescence intensity. In other words, longer irradiation of Ar laser caused the reduction of fluorescence intensity of DCF. So, we decided to irradiate Ar laser every a minute to detect fluorescence shown in Fig. 1 (a).

B. Image analysis

In order to observe active oxygen derived from an activated single monocyte, DIC and fluorescence images were acquired by CLSM. The experiment consisted of the following four conditions.

(a) fluorescence derived from a monocyte added ZnCP-III just before measurement and with HeNe laser irradiation
(b) fluorescence derived from a monocyte only added ZnCP-III just before measurement
(c) fluorescence derived from a monocyte only with HeNe laser irradiation
(d) fluorescence derived from a monocyte with no treatment

Fig. 3 shows typical example of DIC and fluorescence images acquired by CLSM. The images were acquired every a minute of a monocyte added ZnCP-III just before measurement and with HeNe laser irradiation following the protocol given in Fig. 1 (a). The increase of fluorescence intensity indicates that H_2O_2 was generated in a monocyte.

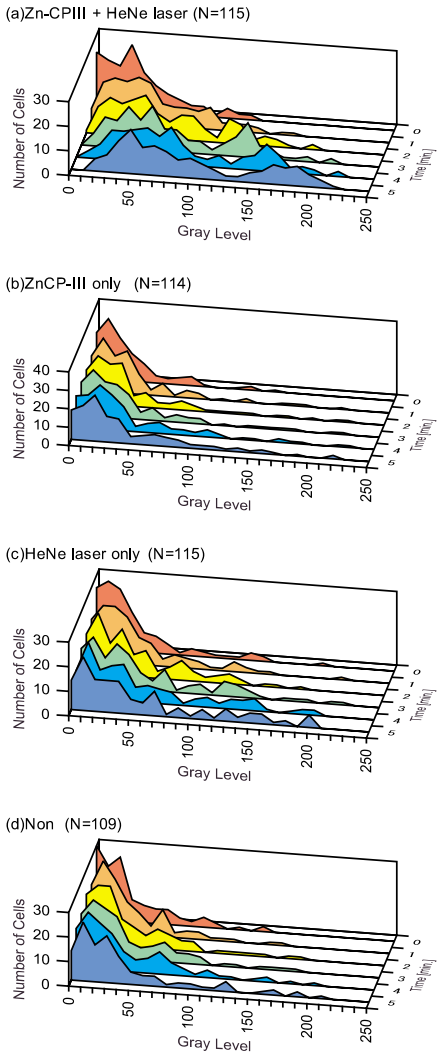

Fig. 4. Fluorescence distribution of monocytes under abovementioned four conditions.

C. Distribution of fluorescence intensity

Fluorescence distribution of monocyte under the abovementioned four conditions is shown in Fig. 4. The values were calculated from acquired images of monocyte. The fluorescence intensity was expressed as gray level, which is graded from 0 to 255. In addition, the intensity was averaged eight times respectively, and reduced to the background intensity level. Only the condition (a) caused fluorescence distribution move to increase, while no change of distribution was observed in other three conditions in particular. Change of relative fluorescence intensity is shown in Fig. 5. The intensities were normalized by those of the first measurement performed before irradiation of HeNe laser($t = 0$). Welch t test for unpaired samples was used to determine the significance of differences. There was a significant difference between the condition (a) and other three conditions.($p < 0.05$)

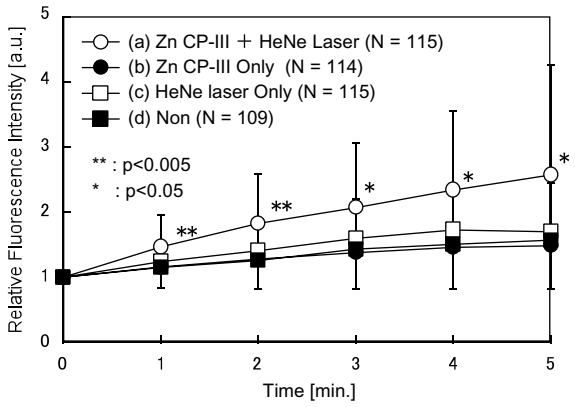

Fig. 5. Change of relative fluorescence intensity. The intensities were normalized with those before the irradiation of HeNe laser(t = 0).The data are expressed as the mean ± S.D. for four separate experiments with above-mentioned number of cells analyzed for each experiments.

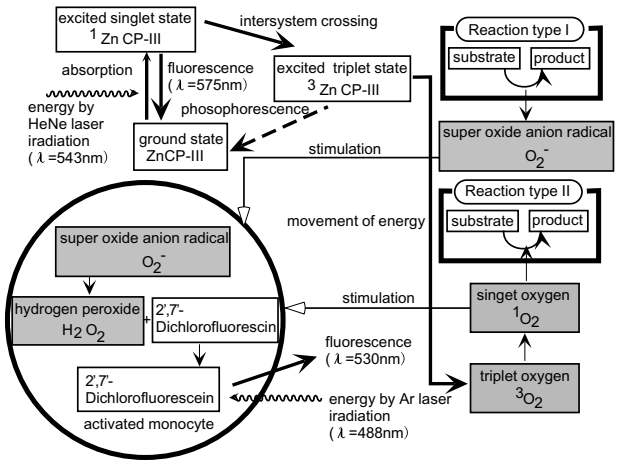

Fig. 6. The process of photochemical reaction and activation of monocytes.

IV. Discussion and conclusion

DCFH-DA was first described by Kenston and Brandt in 1965 [5]. This compound is stable and no fluorescent originally. It can be activated by alkaline hydrolysis to nonfluorescent 2',7'-dichrolofluorescin ; DCFH. DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein ; DCF in the presence of hydrogen peroxide, and the reaction is markedly enhanced by peroxidase. This reaction has been employed to measure the hydrogen peroxide released by PMN. But, this way of quantification called DCFH fluorescence method has ever been used in flowcytometry. So, we analyze DCFH fluorescence image to quantify hydrogen peroxide as a single cell level.

The process of photochemical reaction and activation of monocytes is shown Fig. 6. Zn CP-III is irradiated by HeNe laser and absorbs the energy. Then it is excited from ground state to excited singlet state ;^{1}Zn CP-III. ^{1}Zn CP-III is going to return to a stable state because of high-energy condition and the process is classified into two types. One is the process in which ^{1}Zn CP-III returns to ground state with release of energy as fluorescence. Another is the process with the phenomenon called intersystem crossing. ^{1}Zn CP-III transferred to excited triplet state ;^{3}Zn CP-III. ^{3}Zn CP-III gives an oxygen molecule (triplet oxygen ; $^3O_2$) the energy and returns to ground state. $^3O_2$ turns to singlet oxygen ; $^1O_2$. $^1O_2$ is generated from the reaction called type II in this way, and super oxide anion radical ; O^{2-} is generated from the reaction called type I. This two kinds of active oxygen stimulate monocytes. The activated monocytes, this time for themselves, generate active oxygen and H_2O_2 among them is made visibility by Ar laser irradiation to detect DCF fluorescence. Takahashi et al. demonstrated little permeability of biological membranes to O^{2-}[6]. Considering significantly short diffusion distance and lifespan of the two kinds of active oxygen,they cannot pemeate membrane of monocyte. So,we can conclude that H_2O_2 visualized by DCF fluorescence is derived from O^{2-} generated by monocyte.

Fig. 3 shows the increase of fluorescence intensity of monocyte. The result indicates that H_2O_2 was generated in a monocyte with stimulation by active oxygen due to photochemical reaction. Fig. 4 shows DCF fluorescence intensity, which derived from a monocyte added ZnCP-III just before the measurement and with HeNe laser irradiation ;condition (a), only increased. Fig 5 shows that there was a significant difference between the condition (a) and other three conditions ($p < 0.05$). In addition, whether Zn CP-III is incorporated in a monocyte is investigated by detecting Zn CP-III fluorescence with HeNe laser excitation. The fact that Zn CP-III do not exist in a monocyte during the measurement is confirmed. So, the increase of DCF fluorescence intensity was caused by not photochemical reaction but an activated monocytes.

In conclusion, we confirmed that monocytes are activated by active oxygen caused by photochemical reaction. The result indicates that activated monocytes in this way are concerned with VSD effect and can be one of the factor that thrombus and blood coagulation occurs when PDT is operated.

References

[1] Y. Imazeki, T. Nagao, E. Sekizuka, C. Oshio, and H. Minamitani, "The analysis of mechanism of platelet thrombus formation with photodynamic therapy," Proceeding of the 26th Japan Society for Microcirculation Annual Meeting, pp.69, 2001.

[2] D. A. Bass, J. W. Parce, L. R. Dechatelet, P. Szejda, M. C. Seeds, and M. Thomas, "Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation," J. Immunol., vol.130, no.4, pp.1910-1917,1983.

[3] T. Nagao, Y. Imazeki, H. Minamitani, E. Sekizuka, and C. Oshio, "Evaluation of photobleaching characteristics of a photosensitizer in a single cell using confocal laser scanning microscope," Bioimages, vol.8, no.2, pp.65–72, 2000

[4] T. Nagao, T. Iino, A. Saito, T. Yamamoto, K. Aizawa, and H. Minamitani, "Measurement of active oxygen from photosensitizer pumped by Q-Switched Nd:YAG laser," T. IEE Japan, vol.119-A, no.3, pp.261–266, 1999.

[5] A. S. Kenston and R. Brandt, "The fluorometric analysis of ultramicro quantities of hydrogen peroxide," Anal. Biochem., vol.11, pp.1–5, 1965.

[6] M. Takahashi and K. Asada, "Superoxide Anion Permeability of Phospholipid Membranes and Chloroplast Thylakoids," Arc. Biochem. Biolhys., vol. 226, no23, pp.558–566, 1983.